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Homotypic interaction and multimerization of nucleocapsid protein of tomato spotted wilt tospovirus: Identification and characterization of two interacting domains

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ABSTRACT **The nucleocapsid protein (N) of tomato spotted wilt tospovirus (TSWV) plays a central role in the viral life cycle. With the aid of the yeast two-hybrid system and surface plasmon resonance analysis, homotypic interaction and multimerization of the N protein was detected. Analysis of deletion mutants identified two binding regions in the protein, located at the N terminus (amino acids 1–39) and the C terminus (amino acids 233–248), respectively, implying a "head-to-tail" interaction of the N terminus with the C terminus to form a multimeric chain. Further characterization of the binding domains was performed by site-directed mutagenesis. Two phenylalanines (F242 and F246) highly conserved in the N proteins within the *Tospovirus* genus were shown to play a crucial role in the interaction.**

Tomato spotted wilt tospovirus (TSWV) is the type member of the *Tospovirus* genus, the only plant-infecting genus of the arthropod-borne family *Bunyaviridae.* Tospoviruses have a very broad host range encompassing more than 650 plant species belonging to 70 families, including many crops and ornamentals. Losses to world agriculture are estimated to be more than US$1 billion per year (1).

Molecular biological analysis has confirmed that TSWV is a typical member of the *Bunyaviridae.* The tospoviral particle consists of a core of nucleocapsids in which the genomic single-stranded RNA (ssRNA) molecules are tightly associated with nucleocapsid (N) proteins and a few copies of the L protein. These nucleocapsids are surrounded by a lipid membrane carrying two glycoproteins. TSWV has a tripartite genome consisting of three RNA species called L, M, and S, encoding structural and nonstructural proteins in a negative or ambisense orientation. Despite the fact that the TSWV genome was sequenced several years ago (2–4), knowledge of the biological function of the viral proteins is still very poor. One of the reasons for this lack of information is the limited availability of reverse genetics for *Bunyaviridae*; so far reverse genetics has been developed only for Bunyamvera virus (5). The TSWV L RNA encodes a large polypeptide of 2,875 amino acids proposed to be the viral RNA-dependent RNA polymerase (6). The M RNA codes for the putative movement protein NSm and for a glycoprotein precursor that is processed to the spike proteins G1 and G2 involved in thrips transmission. The S RNA encodes the nonstructural protein NSs with unknown function and the N protein, the main constituent of the TSWV nucleocapsid (1).

Several functions have been established for nucleocapsid proteins of enveloped ssRNA viruses. In general they are basic proteins with nucleic acid binding capacities. Recently, Richmond *et al.* (7) showed that the TSWV N protein nonspecifically binds ssRNA and characterized multiple RNA binding domains. The NP protein of influenza virus (*Orthomyxoviridae*) binds the viral RNA, melts secondary structures, and is in this way involved in the regulation of transcription (8). Direct interaction of the influenza virus NP protein with the polymerase proteins that may be important for regulating the switch of viral RNA synthesis from transcription to replication has been demonstrated by Biswas *et al.* (9). The nucleocapsid protein of hepatitis C virus (*Flaviviridae*) is a multifunctional protein that multimerizes, binds to cell membranes and ribosomes, and has an effect on transcription factors such as c-Myc and c-Fos (10–12). Homotypic interaction and multimerization often is a necessary prerequisite of nucleocapsid proteins for packaging and protecting the viral genome. For measles virus (*Paramyxoviridae*) the nucleocapsid protein shows binding to the viral RNA and associates with the polymerase, thus regulating replication and assembly of nucleocapsids (13). With *Bunyaviridae* it has been shown that the N protein of Rift Valley fever virus, a human- and animal-infecting phlebovirus, acts as an essential factor for the activity of the viral RNA polymerase (14).

These data on nucleocapsid proteins also suggest multiple functions of the TSWV N protein. To fulfill the proposed functions, the following considerations for protein–protein interactions concerning the TSWV N protein are made:

Homotypic interactions leading to multimerization may be a prerequisite for a specific interaction with the viral genomic RNA.

A contact to the glycoproteins can be assumed, helping to organize assembly and budding (15).

There may be interaction of the N protein with the tubular structures formed by the NSm protein, the putative TSWV movement protein (16).

Interaction with the viral RNA polymerase is postulated to regulate its mode of action and facilitate association to the nucleocapsid.

In this study we used the yeast two-hybrid system and surface plasmon resonance (SPR) to provide evidence for a homotypic interaction *in vivo* and multimerization of the TSWV N protein *in vitro*. Deletional analysis and site-directed mutagenesis was used to map interaction sites involved in the homo-oligomerization of the protein.

Abbreviations: TSWV, tomato spotted wilt tospovirus; ssRNA, single-stranded RNA; N protein, nucleocapsid protein; SPR, surface plasmon resonance; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; ONPG, *o*-nitrophenyl β-D-galactopyranoside.

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MATERIALS AND METHODS

Recombinant DNA Manipulations. DNA encoding the TSWV N protein was amplified by PCR using clone pTSWV-L3/308 as a template; this clone had been prepared from genomic RNA of TSWV isolate L3 from Bulgaria (17) (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, DSZM no. PV-0182). Recombinant DNA work was performed by using standard methods (18). Plasmids were maintained in *Escherichia coli* strain DH5α (19) or JM 109 (20). The yeast shuttle vectors were pAS2 and pACT2 (21). The full-length clones of the N gene were amplified by PCR using 5′-oligonucleotides bearing a *Nco*I site and 3′-oligonucleotides carrying an *Xho*I site by using the Expand high-fidelity PCR system (Boehringer Mannheim). Deletional and mutational constructs were generated by PCR using site-specific primers. The fragments were cloned into pAS2 and pACT2, except for NΔ223, which was cloned into pAD-GAL4–2.1 and pBD-GAL Cam vectors (Stratagene), respectively. Accuracy of the constructs was confirmed by sequencing.

Western Blot Analysis. Western blotting was performed by SDS/polyacrylamide gel electrophoresis of yeast protein extract subsequently blotted onto poly(vinylidene difluoride) (PVDF) membranes (Novex, San Diego). Protein preparations from yeast were obtained as follows: Transformed yeast cells were grown overnight in 5 ml of the respective synthetic dropout medium. Fifty milliliters of yeast rich medium (yeast extract/peptone/dextrose; YPD) was inoculated with the overnight culture and grown to an OD_{600} of 0.4–0.6. Cells were washed in distilled water and frozen at −70°C. Yeast protein was isolated by cracking the cells in 8 M urea/5% SDS/0.9% 2-mercaptoethanol supplemented with phenylmethylsulfonyl fluoride (17.4 mg/ml), pepstatin A (0.1 mg/ml), leupeptin (0.014 mg/ml), benzamidine (7.24 mg/ml), and aprotinin (0.37 mg/ml). Blotted proteins were immunodetected by using mouse IgG monoclonal antibodies raised against amino acids 1–147 and 768–881 of the GAL4 DNA-binding and activation domains, respectively (CLONTECH). Secondary goat anti-mouse IgG antibodies that were conjugated to horseradish peroxidase were used, and they were detected by taking advantage of ECL Western blot reagents (Amersham).

Yeast Strain, Transformation, and Two-Hybrid Assays. Competent cells of *Saccharomyces cerevisiae* strain Y190 [*MAT***a***, leu2-3,112, ura3-52, trp1-901, his3-200, lys2-801, ade2-101, gal4*Δ*, gal80*Δ*, URA3*::*GAL-lacZ, LYS*::*GAL-HIS3, cyh*^{r}] (22) were prepared by the LiOAc/single-stranded DNA/PEG method (23). Cotransformed cells were plated onto synthetic dropout medium lacking leucine, tryptophan, and histidine and supplemented with 25 mM 3-aminotriazole (Sigma/Aldrich) to investigate interaction of the hybrid proteins. Recombinant hybrid proteins were tested for self-activation and nonspecific protein-binding properties (24). Interactions of two partners were confirmed by a 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) filter staining assay (25). Yeast transformed with empty vector constructs (pAS2/pACT2) were used as a negative control, whereas yeast transformed with pBD-SNF1 and pAD-SNF4 were used as a positive control (26). Additionally, β-galactosidase activity was assayed with *o*-nitrophenyl β-D-galactopyranoside (ONPG; Sigma/Aldrich) by using a protocol modified from ref. 25. Doubly transformed cells were grown overnight, and aliquots containing 10^7 cells [individually counted in a hemocytometer (Fuchs–Rosenthal chamber)] were resuspended in 160 μl of Z buffer (100 mM sodium phosphate, pH 7.0/10 mM KCl/1 mM $MgSO_4$/50 mM 2-mercaptoethanol/0.05% SDS) in a microtiter plate (Greiner, Solingen, Germany) supplemented with 10 μl of $CHCl_3$. After Vortex mixing for 10 min, the suspension was incubated with 30 μl of a 4 mg/ml ONPG solution for 120 min at 30°C. The reaction was stopped by 75 μl of a 1 M Na_2CO_3 solution, cell debris was pelleted, and 135-μl aliquots of the supernatants were transferred to a new microtiter plate. Absorbance at 410 nm was read in a Dynatech MR4000 ELISA Reader and ONPG values were calculated according to 1 unit = $10^3 \times (A_{410}/120$ min). All tests were repeated at least four times, and a blank reaction with no cells and a standard positive control containing yeast cells transformed with SNF1 and SNF4 (26) were included in the assay. Simultaneously an aliquot containing 10^7 cells was dotted onto a membrane and stained with X-Gal as described (25). Plasmid rescue from yeast cells was performed according to ref. 27.

Expression of $(His)_6$-Tagged TSWV N Protein. Heterologous expression of TSWV N protein was performed with the QIAexpressionist system (Qiagen, Hilden, Germany). DNA encoding the N protein was amplified by PCR using pTSWV-L3/308 as a template and a 5′ oligonucleotide primer with a *Bam*HI restriction site and a 3′ oligonucleotide primer with a sequence encoding six histidines and a *Hin*dIII site. The DNA fragments were cloned into an expression vector derived from pQE9, where nucleotides 107–164 were replaced by a *Bam*HI and a *Hin*dIII site. The accuracy of the constructs was verified by sequence analysis. Histidine-tagged N protein was expressed in *E. coli* M15 [pREP4] cells according to the manufacturer's instructions. Protein extraction was performed under native conditions by resuspending the pellet in sonication buffer (50 mM sodium phosphate, pH 8.0/300 mM NaCl) and treating the cells four times for 30 sec with a Branson sonifier 250 Micro Tip. Purified $(His)_6$-tagged N protein was obtained by affinity chromatography on Ni–nitrilotriacetate (NTA)-agarose and eluted with an imidazole gradient (Qiagen). The protein was then dialyzed against 50 mM sodium phosphate, pH 7.6/150 mM NaCl. Integrity of protein was tested by SDS/PAGE and by Western analysis using antibodies raised against N protein of TSWV isolate BR-01 (anti-BR01-IgG; Loewe Biochemica, Otterfing, Germany) in a standard protocol (18).

SPR Analysis. SPR is a method to investigate interaction between macromolecules. The interaction is recorded in refraction units (RU) detecting the change of the refractive index near the sensor surface. Purified $(His)_6$-tagged N protein was diluted to 50 mg/ml in 10 mM sodium phosphate buffer, pH 6.0/150 mM NaCl and coupled to the dextran-modified gold surface of a SA5 sensor chip by using amine coupling chemistry according to the manufacturers manual (Pharmacia Biosensor, Freiburg, Germany). A $(His)_6$-tagged N protein solution of 5 mg/ml in HBS (150 mM NaCl/10 mM Hepes, pH 7.4/5 mM $CaCl_2$/3.4 mM EDTA/0.005% surfactant P20) was injected onto the chip at a flow rate of 5 ml/min at 25°C, and subsequently the chip was rinsed with HBS. Binding kinetics were determined in comparison to an untreated reference flow cell. To check for nonspecific protein binding, BSA was used as a negative control, either injected with the solution (2 mg/ml) or coupled to the sensor chip.

Size-Exclusion Chromatography. The oligomeric state of purified recombinant expressed N protein was analyzed by size-exclusion chromatography using a precalibrated Sephacryl S-200 HR column (buffer: 150 mM sodium chloride/50 mM sodium phosphate, pH 7.6; flow rate 0.5 ml/min).

RESULTS

Wild-type TSWV N Protein Revealed Homotypic Protein Interaction *in Vivo* in the Yeast Two-Hybrid System. Homotypic interaction of TSWV N protein was detected when yeast strain Y190 was transformed with pAD-N and pBD-N and monitored for histidine prototrophy and β-galactosidase activity (Fig. 1). Protein expression had been verified by Western analysis (data not shown) and no nonspecific DNA-binding or transcriptional activation was found (Fig. 1). Integrity of the N

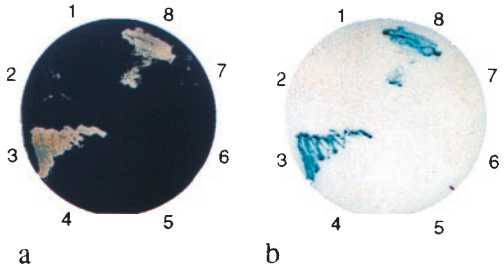

FIG. 1. Cells were plated onto selective medium lacking histidine and supplemented with 25 mM 3-aminotriazole (*a*) and tested for β-galactosidase activity by using X-Gal on a filter lift assay (*b*). Cells were transformed with the following: 1, pBD-N only; 2, pAD-N only; 3, pBD-SNF1/pAD-SNF4; 4, pAS2/pAD-N; 5, pBD-N/pACT2; 6, pBD-SNF1/pAD-N; 7, pBD-N/pAD-SNF4; or 8, pBD-N/pAD-N.

sequences was confirmed by rescuing the plasmids from yeast cells and sequencing.

SPR Analysis Shows Multimerization of N Protein. Wild-type N protein was expressed with a C-terminal $(His)_6$ tag and was purified by using Ni-NTA affinity chromatography. Recombinant protein could be detected at the predicted size in SDS/PAGE, and integrity of $(His)_6$-tagged protein was demonstrated by using an N-specific antibody (data not shown). Evidence for homopolymerization of the N protein in solution has been obtained by size exclusion chromatography. N protein was detected mainly in fractions indicating a molecular mass of >200 kDa. Only a small proportion of the protein was detected as tri-, di-, and monomers (data not shown). Experiments with SPR indicate that interaction of TSWV N protein in solution is a continuous process leading to multimers or aggregates of increasing molecular mass. The protein was coupled to the surface of a sensor chip which was alternately rinsed with a 30 nM protein solution and the respective buffer for 180 sec. Binding was scored in comparison to an untreated reference flow cell. No nonspecific protein binding was found when BSA was used as a negative control. Injection of N protein resulted in a continuously increasing response (Fig. 2). Rinsing of the sensor chip for 180 sec revealed no decrease of the signal, indicating that no dissociation of the bound N protein occurred. Further addition of N protein could again be monitored in a linear increase of the surface resonance (Fig. 2). The continuously increasing signal without saturation indicates further aggregation of the N protein. This was supported by the finding that the slope of the signal was linearly dependent on the concentration of the injected protein (data not shown).

Deletional Analysis of TSWV N Protein Reveals N- and C-Terminal Interaction Sites Responsible for Protein Interaction in the Yeast Two-Hybrid System. N- and C-terminal deletion mutants of TSWV N protein were generated. Constructs were tested for changes in their capability to show homotypic interaction or interaction with wild-type N protein. Histidine prototrophy and blue color in an X-Gal assay was scored as a positive result. To quantify the strength of the interaction, doubly transformed cells were grown in selective medium and aliquots containing 10^7 cells were subjected to a β-galactosidase activity test using ONPG.

Two C-terminally truncated deletions were generated which are indicated as CΔ10 (encoding amino acids 1–248) and CΔ26 (amino acids 1–232) (Fig. 3). CΔ10 was scored positively when tested for homotypic interaction, whereas CΔ26 was not. However, both constructs were found to interact with wild-type N protein (Fig. 4). Quantification with an ONPG assay confirmed the results and differentiated the data: The homotypic interaction of wild-type N resulted in a weak signal (0.167 unit), whereas the interaction of CΔ10 and CΔ26 with wild-type N gave higher values in an ONPG assay (0.277 and 4.027 units, respectively). No significant interaction was observed for the homotypic CΔ26 interaction (0.008 unit) (Fig. 4). From three N-terminal deletions termed NΔ22 (encoding amino acids 23–258), NΔ39 (amino acids 40–258), and NΔ223 (amino acids 224–258), respectively, only NΔ22 showed positive homotypic interaction (ONPG value: 0.835 unit) whereas the other mutants scored negatively. Nevertheless, interaction with wild-type N protein was detectable for all N-terminal deletions scoring with high ONPG values (see Fig. 4). When a doubly truncated construct NΔ22CΔ26 (encoding amino acids 23–232) was tested, again no homotypic interaction could be detected, but interaction with wild-type N protein could be observed. The strength of this interaction was scored at 0.385 unit.

These results suggest that both a C-terminal region (amino acids 233–248) and an N-terminal region (amino acids 23–39) are essential for the homotypic protein interaction. The proposed interaction site at the C-terminal region of TSWV N protein was further characterized by point mutations and combination of these with NΔ22 (F242A, F246A, F242/246A, NΔ22-F242A, NΔ22-F246A, NΔ22-F242/246A; Fig. 3). The two phenylalanines F242 and F246, located in the C-terminal region of the N protein mentioned above, were found to be highly conserved within the core proteins of tospoviral species and therefore were chosen for site-specific mutagenesis and mutated to alanines (Fig. 5). When the constructs F242A or F246A were tested, homotypic interaction and interaction with wild-type N protein were detected at similar levels. F242/246A showed strongly reduced homotypic interaction (0.124 unit) but interactions with wild-type N protein could be observed at the same level as above (Fig. 6). When NΔ22-F242A, NΔ22-F246A, and NΔ22-F242/246A were tested no homotypic interaction could be observed, although for all three constructs considerable interaction with wild-type N protein could be measured (Fig. 6).

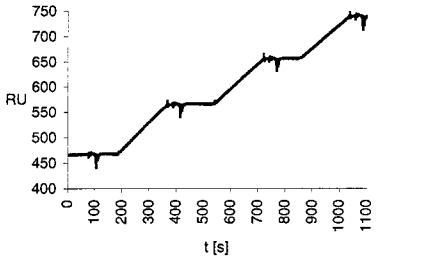

FIG. 2. SPR of the interaction of TSWV N protein subjected to a SA5 sensor chip that had been preloaded with N protein. The sensor chip was alternately rinsed with a 30 nM protein solution (at 190, 550, and 860 sec) and the respective buffer (at 0, 370, and 730 sec).

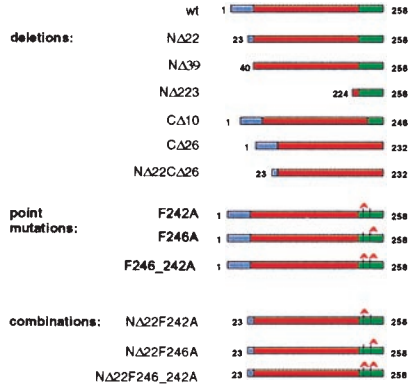

FIG. 3. Deletional and mutational constructs generated for the yeast two-hybrid system to investigate homotypical interaction and interaction with wild-type N protein. Numbers of amino acid residues are indicated. A red A indicates schematically where alanine was substituted for phenylalanine.

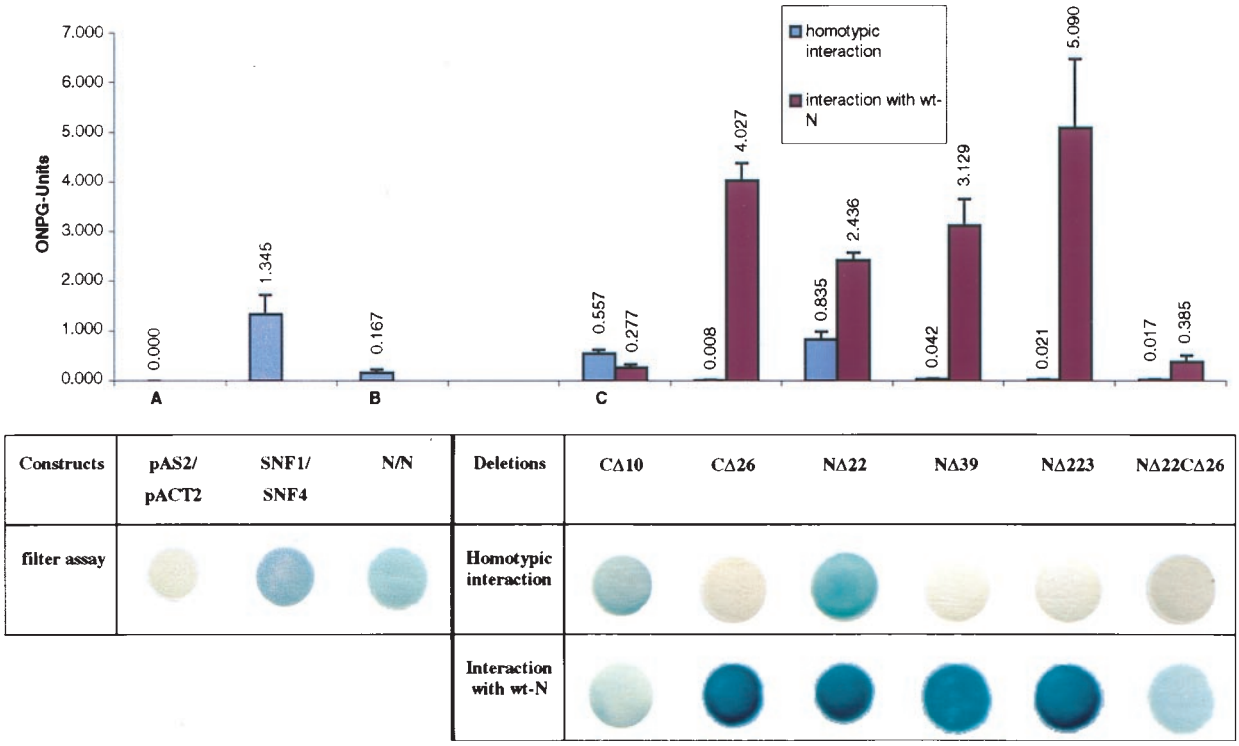

FIG. 4. Homotypic interaction of wild-type N protein (*B*) and interaction of C- and N-terminally truncated proteins (*C*). The strength of the interaction was determined by assaying the β-galactosidase activity with ONPG. Aliquots containing 10^7 cells of an overnight culture were spotted onto a membrane and stained with X-Gal. Empty vector constructs and SNF1/SNF4 were used as controls (*A*).

Additionally, combinations of deletional and mutational constructs were used to verify the role of the two interaction sites (Fig. 6). NΔ223 was found to strongly interact with CΔ26, whereas no interaction was found between NΔ223 and NΔ39. When CΔ26 was used in combination with F242A, F246A, and F242/246A, strong interaction between CΔ26 and F242A was detected (2.523 unit), whereas interaction of CΔ26 and F246A was found to be weaker (0.369 unit). Interaction of CΔ26 with F242/246A was further decreased and scored at 0.083 unit.

DISCUSSION

The N protein of TSWV constitutes the main part of the nucleocapsid, the infectious subviral particle of TSWV, which contains the viral genomic RNA and a few copies of an RNA-dependent RNA polymerase (1). Mainly from data on capsid proteins of other enveloped viruses, an important role in the structural organization of the viral life cycle can be ascribed to the TSWV N protein. The primary function of viral core proteins, based on their RNA-binding capability, is assumed to provide a protective shell for the viral genome inside. A nonspecific ssRNA binding capacity of the TSWV N protein has been demonstrated recently (7). Furthermore, a direct interaction with the viral RNA polymerase can be postulated to facilitate its association to the nucleocapsid (28) and possibly mediate transcription and replication. Contact with TSWV glycoproteins is suggested to facilitate assembly and budding of the viral particle (15). There may also be interaction of the TSWV N protein with the tubular structures formed by the NSm protein, the putative movement protein, to organize cell-to-cell movement of the nucleocapsid (16). In addition, Steinecke *et al.* (29) discuss a regulatory function of the N protein during the early events of TSWV transcription and replication. In a similar context Lopez *et al.* (14) have demonstrated that the N protein is indispensable for the activity of the viral RNA-dependent RNA polymerase from Rift Valley fever virus, a phlebovirus within the *Bunyaviridae*.

A necessary prerequisite to fulfill these functions is a capability of the N protein to take part in multiple protein–protein interactions. With regard to the large family of mainly animal- and human-infecting *Bunyaviridae,* no molecular data on the underlying structural requirements of the N protein have been available so far.

We have shown that the TSWV N protein is able to participate in homotypic interactions *in vivo* and forms homomultimeric structures *in vitro* in the absence of viral genomic

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                                        *  *
TSWV-L3          217 AILSSSNPNAKGSIAMEHYSETLNKFYEMFGV----------------KKQAKLTELA 258
TSWV-BR-01       217 .............V.................----------------......A... 258
GRSV-SA-05       217 K..............DY..DN.E.......----------------..E..IAGV. 258
TCSV-12851       217 T....C.............H.D...A....----------------R.E..ISGV. 258
INSV-NL-07       221 D..KACS.K...LA..D..K.G.TSI.S..NA----------------TIDFGKNDSI 262
INSV-Beg         220 D..KACS.K...LA..D..K.G.TSI.S..NA----------------TIDFGKNDSI 261
WSWV-W           221 K...E...GTAS..SLKK.EDQVKYMDRV.SANLSVDDYGDHSKK-SS.PSTS.E--V 275
PBNV-40687       221 KL..E...GTAS..SLKK.D.QVKYMDKA.SASLSMDDYGEHSKKKSS.AGPS.E--L 275
IYSV-60456       221 K..TDCT.GTS.AASLIKFN.QIKILEGA..KI--VEDIGESSK---P.TPS.KDRYN 273
consensus              L    P                    F
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FIG. 5. C-terminal part of tospoviral N proteins. Multiple sequence alignment using PILEUP [Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, WI]. Dots indicate amino acid residues identical to those in TSWV-L3; asterisks indicate residues F242 and F246 that have been mutated to alanine; and hyphens denote gaps. GenBank, EMBL, PIR, Swiss-Prot accession numbers: TSWV-L3 (tomato spotted wilt virus), P26001; TSWV-BR-01 (tomato spotted wilt virus), 420617; GRSV-SA-05 (groundnut ring spot virus), P36294; TCSV-12851 (tomato chlorotic spot virus), P36293; INSV-NL-07 (impatiens necrotic spot virus), 60490; INSV-Beg (impatiens necrotic spot virus), 310991; WSWV-W (watermelon silver mottle virus), 1019831; PBNV-40687 (peanut bud necrosis virus), 881480; and IYSV-60456 (iris yellow spot virus), 2228768.

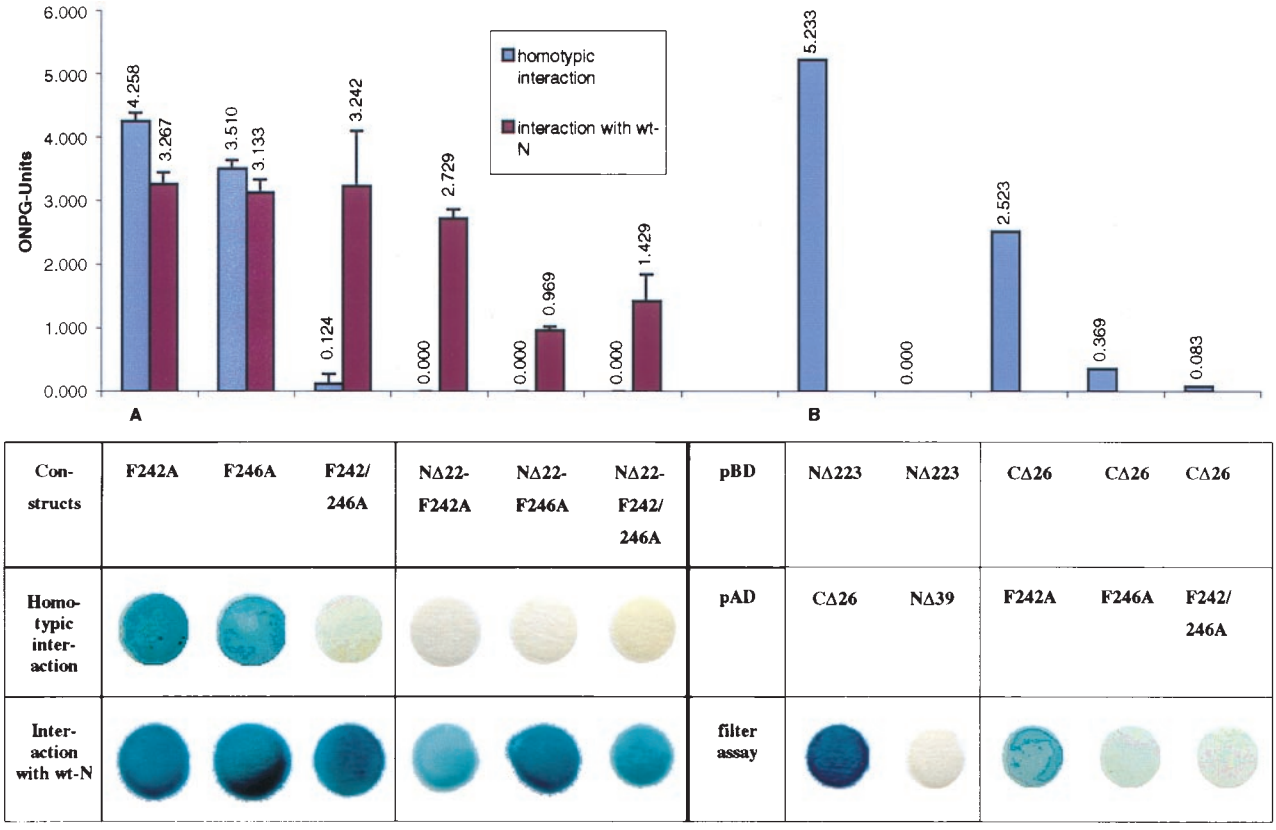

FIG. 6. Interaction of full-length and N-terminally truncated proteins in which F242 and/or F246 has been mutated to alanine. Constructs were tested for homotypic interaction and interaction with wild-type N protein (wt-N) (*A*). The strength of the interaction was assayed by a β-galactosidase activity test using ONPG. Additionally, interaction between a set of deletional or mutational constructs was determined (*B*).

RNA. Multimerization of nucleocapsid proteins has been shown for many enveloped ssRNA viruses such as human immunodeficiency virus, Moloney murine leukemia virus, hepatitis C virus, and Sendai virus (12, 30–32).

Owing the fact that nothing is known about the mechanism of how the TSWV N protein assembles into a nucleocapsid structure, the experimental proof of N protein self-interaction might provide a basis for a better understanding of nucleocapsid formation and the role of the N protein in transcription and replication, since the latter is thought to be regulated by the monomeric/multimeric state of the N protein. One could further speculate that the multimeric state of TSWV N protein may be of significance for the specific recognition and correct binding of the viral RNA. This in turn may be obligatory to mediate the accessibility of the RNA for the RNA-dependent RNA polymerase.

We have dissected the homomultimerization at the molecular level by analyzing deletion mutants and performing site-directed mutagenesis. The deletion analysis identified two regions of the protein involved in the homotypic interaction. While a truncated protein lacking the last 10 amino acids is similar to the full-length protein with regards to its capability to form homodimers or to interact with wild-type N protein, deletion of the C-terminal 26 amino acids (CΔ26) abolishes the capacity to participate in a homotypic interaction. Nevertheless, this truncated protein is able to interact with wild-type N protein, indicating an interaction of the C-terminal region with a second region within the protein. This finding is further supported by the fact that a small C-terminal fragment of the N protein, comprising only the last 39 amino acids (NΔ223), is able to interact not only with the wild-type protein but also with the truncated CΔ26.

Investigating deletions of the N terminus revealed a second binding domain formed by a region within the first 39 amino acids of the N protein. A deletion construct lacking the first 22 amino acids is still able to participate in homotypic interaction. Nevertheless this deletion affects part of the N-terminal binding domain, weakening the interaction, as discussed below. Deletion of 17 additional amino acids completely eliminates self-interaction. However, this truncated protein NΔ39 retains the capability to interact with the wild-type N protein. Additionally, NΔ39 interacts with the C-terminal deletion mutant CΔ26 (data not shown). The fact that no interaction of the short C-terminal fragment NΔ223 with NΔ39 could be detected further supports the view of an N-terminal binding domain within the first 39 amino acids.

From these results a "head-to-tail" organization of the homotypic interaction of the TSWV N protein can be postulated, involving an N-terminal region (amino acids 1–39) and a C-terminal region (amino acids 233–248) of the protein. This fits the general idea that multimerization requires at least two distinct binding regions. The fact that deletion of either of the two domains abolishes the ability to form homodimers in the yeast two-hybrid system let us assume that there are no other interacting domains.

Interestingly, the strength of the interactions between the deletion mutants and the wild-type N protein as measured by a β-galactosidase assay using ONPG is stronger than the homotypic interaction of the wild-type N protein. Since it is not very likely that both the C-terminal and the N-terminal regions of the N protein perturb the interaction, these results appear as a contradiction. This might indicate a problem of the two-hybrid system in monitoring homotypic interactions. The fact that interaction of the binding domain fusion proteins alone (BD-N·BD-N) or the activation domain fusion proteins alone (AD-N·AD-N) is possible might impede the correct reconstitution of the GAL4 transcription factor (BD-N·AD-N). A reduction or elimination of the ability to participate in

homotypic interaction might thus lead to an apparent increase in interaction strength, as a consequence of a diminished competition between BD-N·AD-N with BD-N·BD-N and AD-N·AD-N.

To get further insight into the molecular basis of the homotypic interaction we investigated the role of individual amino acids located in the C-terminal binding region by site-directed mutagenesis. Protein sequence comparison of the N protein of different tospoviruses revealed two conserved amino acids in this region: phenylalanine 246 is conserved within all tospoviruses and phenylalanine 242 is conserved within the members of serogroups I and II [TSWV, GRSV, and TCSV (33)]. These two amino acid residues were mutated to alanine either individually or in combination. The three mutant proteins were able to interact with the wild-type N protein. Characterization of the homotypic interaction of these mutant proteins revealed an involvement of both amino acids in binding. As compared with the homotypic interactions of the mutants mentioned above, the interaction strength of the homotypic interaction of F242A and F246A, respectively, is in the same range of magnitude, whereas the strength of the homotypic interaction of the double mutant, F242/246A, is dramatically decreased. The fact that both phenylalanines are involved in the interaction is further supported by quantitative data from the interaction studies between these mutants and the C-terminal mutant CΔ26. The two amino acids contribute to the strength of the interaction to different degrees. The mutation of phenylalanine-246 leads to a weaker binding than mutation of phenylalanine-242, as compared with the interaction of wild-type N protein with CΔ26. Combination of the two point mutations (F242/246A) virtually abolishes the capacity to interact with CΔ26.

The combination of the point mutations and the deletion NΔ22 leads to a significantly decreased interaction strength with wild-type N protein and a complete loss of homotypic interaction, indicating a participation of the N-terminal 22 amino acids in the binding domain.

In conclusion, we have demonstrated two binding domains in the TSWV N protein promoting the homotypic interaction and multimerization. One is located at the C terminus, the other at the N terminus, supporting the view of a head-to-tail organization of the interaction. Furthermore we have identified two conserved phenylalanines that play a crucial role in the homomultimerization, indicating that the multimerization is based on hydrophobic interactions.

We consider that the analysis of the molecular basis of the homotypic interaction and of other interactions with N protein will be the starting point for a better understanding of the control of transcription, replication, and assembly of TSWV. From vesicular stomatitis virus, HIV, Sendai virus, and other viruses it is known that there are viral proteins possessing overlapping binding domains involved in multimerization, RNA binding, or contact to other viral or host proteins (34–36). Hence there is reason to assume that the mutual influences or interdependence of different or overlapping binding regions may be a fundamental means to regulate various steps of the viral life cycle. Recent findings that there are multiple RNA-binding domains, located at the N terminus and the C terminus of the TSWV N protein, respectively (7), which overlap the two domains responsible for homotypic interaction, support the idea of an interdependence of these two properties of the N protein. Further investigation of the structural basis for both, the RNA binding and the various protein–protein interactions of the TSWV N protein, is needed to better understand the mechanisms of nucleocapsid formation, assembly, and regulation of transcription and replication of TSWV.

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